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Wnt genes encode a large family of secreted signaling molecules related to the *Drosophila* pattern regulating gene *wingless*. Several members of the *Wnt* family have been implicated in mammary tumorigenesis suggesting that Wnt-activated pathways are a normal component of mammary gland development. Consistent with this hypothesis we have demonstrated that seven *Wnt* genes are expressed in the mammary gland at different stages of development. We have started to dissect their activities genetically. One of these, *Wnt5b*, was mutated by gene targeting. However, *Wnt5b* mutant females exhibit normal mammary gland structure and function. A second member, *Wnt4* was investigated by mammary epithelial grafting and found to play a role in ductal branching with a phenotype similar to progesterone receptor mutants. Further analysis supports a linkage between these pathways. We also determined that *Lmx1b*, a target of Wnt-signaling in the limb, is required at embryonic stages for development of the mammary gland. Finally, we have developed strains of mice which will allow mammary epithelial specific CRE-mediated modification of gene activity which should be a useful resource for the scientific community.

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FOREWORD

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5. INTRODUCTION

Nature of problem/Background of previous work.

The mammary gland develops from an epithelial outpocketing of the ventral ectoderm at 11dpc in the mouse embryo [1-4], in response to an initial inductive signal from the underlying mesenchyme [5]. In the female mouse embryo, there is little change in the primary bud over the next four days. However, in the male, mesenchyme surrounding the epithelium condenses from day 14 and this is followed by a rapid necrotic degeneration of the epithelial rudiment. Tissue recombination experiments have convincingly demonstrated that this process is dependent upon testosterone [6], and correlates with the acquisition of testosterone receptors by the mammary mesenchyme [7], which occurs in response to epithelial derived signals [8], and the initial production of testosterone by the embryonic testes. In addition to testosterone responsiveness, the epithelium also induces estrogen responsiveness but, at this stage of development, there appears to be no in vivo role for estrogen [1]. Thus, by the end of this resting period, which is characterized by the appearance of the mammary bud (16.0 dpc), the female mammary gland is poised for further development whereas the male gland is destroyed.

From 16.0dpc to 2dpp, the mammary epithelium extends as the primary mammary sprout into the mesenchyme reaching the fat pad precursor where epithelial branching is initiated. The trigger for elongation of the mammary epithelium is not known, however outgrowth follows the resumption of proliferative activity in the epithelium. The trigger for epithelial branching clearly resides in the mesenchyme of the fat pad precursor. Epithelial morphogenesis, which forms slim epithelial ducts with secondary lateral buds, is specific to the fat pad mesenchyme [9]. Growth into other sources of mesenchymal tissue *in vitro* , e.g. salivary mesenchyme, produces epithelial outgrowths typical of the organ from which the donor mesenchyme was removed. [10]. Thus, shortly after birth in the mouse, and in other mammals [2], the female mammary gland consists of a primary duct connecting with a rudimentary branched epithelium within the presumptive fat pad.

From the period after birth to approximately 4 weeks pp there is very limited growth of the branching epithelium of the mouse mammary gland. However, at 4 to 6 weeks, coupled with the acquisition of sexual maturity, there is a period of extensive cell growth in which the epithelial ducts elongate and branch, extending throughout the fat pad. [11,12] Renewed growth correlates with the reappearance of end buds, a monolayer of unspecialized epithelium at the ends of the ducts. The end buds are thought to contain stem cells which generate differentiated ductal epithelium and myoepithelial cells of the gland. Thus, post natal branching morphogenesis is regulated largely at the termini of the ducts, by controlling the proliferative activity of the end buds. End bud activity is in turn dependent upon ovarian hormones, as ovariectomy results in a rapid loss of end buds and cessation of growth [11,12].

After reaching sexual maturity (6 to 8 weeks pp), further ductal development stops until pregnancy is established. At this time, a second extensive period of ductal growth and branching occurs to fill all the remaining interductal space in the fat pad. During the later phase of pregnancy there is an accompanying development of lobuloalveolar epithelium. Along with the morphogenetic changes in the gland during pregnancy, there is a progressive development of secretory epithelium, such that by birth, a fully functional lactogenic epithelium is established. Interestingly, cytodifferentiation of secretory epithelium will occur in vitro in the absence of morphogenesis, indicating that the two processes are not mutually dependent [13]. Finally, on cessation of suckling, there is a massive involution of the mammary gland due to a widespread destruction of epithelial tissue and the cycle of branching morphogenesis is repeated at the next round of pregnancy.

The mammary gland is unusual, with respect to most organs, in that most of its growth occurs in the adult, and that there are cyclical periods of growth and regression. The control of these processes has been extensively studied and compelling evidence exists for complex regulation mediated by systemic hormonal signals, and locally acting peptide growth factors (for review see [11,12]).

The initial observation that ovariectomy leads to a cessation of end bud growth implicated hormones in the control of mammary development. There is an absolute requirement for estrogen for proper development of epithelial branching. Maximal growth also appears to require growth hormone or prolactin [11,12]. However, whether these hormones act directly, or sensitize the epithelium to the action of other factors, is not clear. Lobuloalveolar growth requires, in addition to the above, progesterone which accumulates later in pregnancy. Finally, the onset of lactation correlates with the increase in prolactin and glucocorticoids and a decrease in progesterone [11,12].

Evidence for involvement of peptide growth factors in the regulation of mammary development has come from the direct observation of growth factor expression, and implant and transgenic studies which have manipulated growth factors in the mammary gland. Slow release implants of EGF stimulates local growth of end buds in quiescent mammary epithelium [14], whereas implantation into growing mammary glands causes local inhibition of ductal growth, and a down regulation of EGF receptors [15]. Thus, EGF may have a dual specificity depending upon the particular stage of development. TGF-β1 implants also suppress ductal growth [16] acting specifically on the end buds to inhibit DNA synthesis [17] whereas TGFα stimulates alveolar and ductal growth [18-21].

Additional evidence for peptide factors in growth regulation has come from the analysis of mammary tumors in which growth controls have been uncoupled following expression of genes not normally active in the mammary gland. A number of loci, have been shown to undergo MMTV mediated insertional activation in mouse mammary tumors (for review see [22]). Four of these encode secreted peptide factors, *Wnt-1* [23] and *Wnt-3* [24] members of the *Wnt*-gene family, and *FGF-3* [25] and *FGF-4* [26], members of the fibroblast growth factor family. Additional evidence suggests that *Wnt* and *FGF* genes may cooperate in tumor formation as frequently *Wnt-1* and *FGF-3* are co-activated in the same mammary carcinomas [27].

The oncogenic role of *Wnt-1* has been demonstrated by *in vitro* and *in vivo* studies. Transfection of the *Wnt-1* gene into C57MG cells, a primary mammary epithelial cell line, leads to morphological transformation [28,29]. However, these cells do not grow in soft agar or form tumors in syngeneic hosts. In contrast RAC311C cells are rendered morphologically transformed and tumorigenic when transfected with *Wnt-1* [30]. Formal proof of the transforming roles of Wnt-1 has come from transgenic studies which lead initially to hyperplasia, in both the male and female mammary gland, and progress to the formation of adenocarcinomas [31]. As was observed in spontaneously occurring tumors, there is also a synergistic affect of *FGF-3* on *Wnt-1* transformation in the transgenic model [32].

In addition to *Wnt-1* and *Wnt-3*, fourteen additional members of the mouse *Wnt*-gene family have been identified. Human Wnt-2 was isolated serendipitously in a search for the cystic fibrosis gene [33,34]. Like *Wnt-1* and *Wnt-3*, Wnt-2 is implicated in tumorigenesis as it appears to be amplified and highly expressed in some MMTV induced tumors [35]. Amplification appears not to be related to MMTV, but is a novel mechanism which presumably acts in conjunction with MMTV activated genes to transform epithelial cells [35]. *Wnt-3a* was identified on the basis of its close relationship to *Wnt-3* [36], and *Wnt's-4, 5a, 5b, 6, 7a, 7b*, on the basis of a PCR cloning approach [37] which has been successful in identifying *Wnt*-genes in many species, as well as two new mouse members (*Wnt-10* and *11*; A. McMahon, unpublished data).

All Wnt-proteins have several features in common including a putative signal peptide sequence, one conserved glycosylation site, and 20 absolutely conserved cysteine residues. Typically Wnt proteins are 38 to 45kd. Although only *Wnt-1* and *Wnt-2* have been studied, and these analyses have been restricted to cell culture systems, both genes appear to encode poorly secreted glycoproteins with strong affinity for cell surface and/or extracellular matrix [29,38-44]. Thus, it is likely that they are involved in short-range signaling. Functional analyses of several members indicates these important regulatory roles in invertebrate and vertebrate development [reviewed in 45,46].

The observation that *Wnt* expression leads to morphological transformation of mammary epithelial cells *in vitro* and hyperplastic growth *in vivo* indicates that mammary epithelium is responsive to *Wnt* gene products. If, Wnt-proteins act as signals (a conclusion greatly strengthened by studies on the Drosophila Wnt-1 orthologue *wingless*, [46]), then by analogy with other families of peptide signals, it would seem likely that the responsiveness of mammary epithelium reflects the expression of functional Wnt-receptors.

Recent evidence demonstrates that unlike *Wnt-1* and *Wnt-3*, six family members are expressed, and developmentally regulated, during normal adult mammary gland development [47]. Thus, the responsiveness to ectopic expression of *Wnt-1* or *Wnt-3* presumably reflects some modulation of *Wnt*-signaling pathways which normally respond to endogenously expressed Wnt-factors. For example, if Wnts normally stimulate cell growth, ectopic expression of *Wnt-1* or *Wnt-3* may lead to hyperstimulation of a proliferative Wnt-signaling pathway. Conversely, if endogenously expressed *Wnts* suppress proliferative activity, ectopic *Wnt-1* or *Wnt-3* expression may block Wnt-mediated growth suppression, possibly by interfering with receptor function.

The situation is likely to be complex on the basis of our studies of *Wnt*-transcription in the adult mammary gland [47b] . *Wnt-2* expression is very weak and confined to virgin or nonpregnant mice [47b]. Thus although *Wnt-2* causes C57MG cell transformation, its expression does not correlate with proliferative activity. Quite the opposite, it is limited to the quiescent state. *Wnt-5a* and *Wnt-7b* are also expressed at low levels in virgin mice [47]. However, expression extends into mid but not late pregnancy showing decreasing levels of expression despite the large increase in mammary epithelium. In contrast, *Wnt-5b* and *Wnt-6* are expressed at low levels prior to pregnancy and increase considerably to midpregnancy, declining by parturition [47]. Thus, these two members show a better correlation with epithelial expansion. Finally *Wnt-4* expression is uniform from in the virgin gland until late in pregnancy when it rapidly declines [47].

Transformation assays on C57MG cells indicate that several Wnt-members which are normally expressed in the mammary gland are transforming in this assay [48]. Wnt-2, -5b and -7b are moderately transforming, weaker than Wnt-1, 3a and 7a, whereas Wnt-4, 5a and 6 are non transforming. *Wnt-4* and *Wnt-5a* are normally expressed by C57MG cells, thus elevation of endogenous expression several fold does not lead to transformation. These results suggest that hyperplasia *in vivo* may result from inappropriate activation of Wnt-2, Wnt-5b and/or Wnt-7b signaling pathways.

In summary, the data clearly support a model in which normal mammary, epithelial growth is regulated by one or more *Wnt*-genes. They demonstrate that uncoupling of these regulatory pathways leads to hyperplasia [31,49] and adenocarcinomas *in vivo* [31]. However, without a better understanding of the normal spatial expression of Wnt-proteins and their putative receptors, and the transforming activity of the family as a whole *in vivo*, we are not in a position to grasp the full significance of their functions in the normal and transformed mammary tissue, nor the relevance that this family may have to human breast cancer.

Purpose of present work/Methods of approach

As discussed above it is now fifteen years since Nusse and Varmus identified a locus in the mouse associated with the generation of mammary tumors. It is now clear that the associated gene, *Wnt-1*, is one member of a large family of putative signaling molecules which normally regulate embryonic development. Several members have now been implicated in epithelial cell transformation in the mammary gland from the analysis of spontaneously occurring mouse tumors (*Wnt-1, Wnt-3, Wnt-3a*), transgenic experiments (*Wnt-1*) and *in vitro* studies (*Wnt-1, 2, 3, 3a, 5b, 7a, 7b*). Thus, it would appear that hyperplasia, and eventual adenocarcinoma formation, in the mouse mammary gland result when normal growth regulatory pathways which are presumably controlled by Wnt-proteins, are perturbed by deregulated expression of certain Wnt-family members.

Understanding growth control in the mammary gland is essential for designing strategies which will treat mammary tumors. Further, potential growth regulators are likely mediators of mammary transformation, as exemplified by studies on *Wnt*-genes in the mouse, and should thus be examined for contributory roles in human mammary cancer. This proposal set out to examine the normal and oncogenic roles of Wnt protein in the mammary gland of the mouse and human, and to dissect the Wnt-regulatory pathways at the receptor level. Specifically we proposed to address the issue of whether *Wnt*-genes may be involved in human cancers by directly examining expression in mammary tumors using Northern blot analysis. We propose to use transgenic mice to examine the relationship between normal *Wnt*-gene expression and mammary transformation. As Wnt-signaling is most likely a conventional receptor-mediated process, ectopic expression of specific Wnt-signals presumably exerts its effects through one or more receptor pathways coupled to endogenously expressed Wnt-proteins. If so, we should be able to identify a likely candidate pathway by assaying the transforming potential of endogenously expressed Wnt-proteins when their normal regulation is uncoupled, either by ecoptic expression or gene ablation. Moreover, characterizing the normal expression of *Wnt*-genes and their products in relation to the developing mammary gland may provide strong suggestive evidence as to what growth regulatory pathways may be responsive to Wnt-signals. Finally we propose several approaches toward identifying Wnt-receptors which will be an essential step in fully defining Wnt-signaling pathways, and their regulatory function in the mammary gland. Thus, the proposed studies are directly relevant to the issue of the genetic alterations involved in the origin and progression of cancer and the changes in cellular and molecular function which may account for the development and progression of breast cancer.

In summary we proposed five specific goals

1) To determine, using transgenic mice, which if any of the *Wnt*-members normally expressed in the mammary gland are oncogenic when ectopically expressed using an MMTV enhancer construct.

2) To determine the relevance, if any, of *Wnt-5b* in normal gland development by studying mice homozygous for a likely null mutation in the *Wnt-5b* gene.

3) To determine the normal temporal and spatial expression of *Wnt* genes, and their protein products, during embryonic and adult mammary gland development.

4) To use various schemes to attempt to identify other proteins, particularly candidate receptors, which interact with Wnt-proteins.

5) To isolate sequences encoding all of the yet-unidentified human *Wnt*-genes, providing clinicians with a broad array of Wnt-probes which may be important in the analysis of human mammary carcinomas.

6. BODY

Over the period of the award we made substantial changes as a result of ours and others findings in a field that has developed rapidly. This lead to a change in our goals as reported in previous reporting periods.

1) Transgenic analysis of Wnt-mediated oncogenesis

Our studies identified that Wnt10a, Wnt-10b and Wnt-6 are expressed in the mammary epithelium at the time that the mammary buds are formed. This data has been reported previously. None of these is exclusive to the mammary gland at this time. Wnt-10a and Wnt-10b are expressed more widely in a variety of epithelial placode derived structures Dassule and McMahon (1998). Wnt-6 is expressed widely in ectoderm. Thus, it is possible that Wnt-1/3/3a mediated tumorigenesis may occur through a signaling pathway in place to respond to Wnts normally present at this early stage. We proposed to test more thoroughly the tumorigenic role of different Wnts using the MMTV transgenic model. However, in light of the finding that MMTV activation of Wnt-10b leads to mammary tumorigenesis [53] and the strong likelihood that this is the case for Wnt-10a given the strong sequence conservation, we focused our efforts in achieving other goals.

Recommendation for future studies

The failure of mammary gland development exhibited by mutants in Lef-1[54], a transcriptional mediator of Wnt-signaling provides compelling evidence that Wnts are involved at the earliest stages of mammary embryogenesis. Given that such mutants also have defects in hair and tooth development, both sites of expression of Wnt10a/b, it is tempting to conclude that these family members play a redundant role in the regulation of mammary gland development, acting through Lef1. Indeed, evidence from the Wnt-10b knock out indicates defective growth of the mammary epithelium [Dr. P. Leder, personal communication]. Further, the recent linkage of b-catenin to cyclin D1 regulation [55,56] suggests a scenario in which Wnts may directly regulate cell cycle genes in the mammary epithelium. This would be an interesting avenue to explore. The model that Wnts are directly hooked into cyclin control, and through this pathway control of proliferation within mammary tissue, would tie in well with the predicted outcome of deregulated high level expression of Wnts following MMTV insertion, if of course those same Wnts can signal through this pathway.

2) *Wnt-5b* mutant analysis

We succeeded in generating Wnt-5b mutants as discussed in previous reports. However, despite the widespread expression of this member of the family, mutants were viable and showed no mammary phenotype. The mutation inserted a positive selection cassette into the fourth coding exon which was predicted to generate a truncated protein which would lack activity. However, it is now not clear that this would be the case given that some carboxyl truncations of the Drosophila relative, wingless, retain activity [57]. In the absence of a reasonable bioassay to determine whether the truncated protein retained activity we decided that retargetting the Wnt5b locus was beyond the scope of this proposal.

Recommendation for future studies

It seems reasonable to treat Wnt-signaling as a whole in the mammary gland rather than being too preoccupied with any one signal. This is what we have attempted in the following section. Unfortunately the only reliable way of determining whether Wnt-5b plays a role is to unambiguously remove gene function. If this was done, and viable mice without a phenotype were obtained, the results would clearly argue that Wnt-5b has either no role, or a redundant role. However, if there were activity in the originally targeted allele, and all activity is now removed, given the widespread expression of Wnt-5b, the result may be a mouse that dies at an

early stage which would require a third approach to directly address mammary gland development (see below).

3) **Wnt expression in the mammary gland**

Although we have published that several *Wnt* genes are expressed during mammary gland development in the adult [48], we do not know in which cell types, nor the spatial details. This information is important. Growth and branching morphogenesis are primarily regulated at the end buds. Thus, any growth stimulatory or growth repressive action of a Wnt member is likely to act on this aspect of the epithelial network. As the available evidence suggests that Wnts are short range factors, we would therefore anticipate that some Wnt members will be locally distributed either in the stroma surrounding the end buds, or perhaps in the end bud themselves, and their expression would be predicted to change dramatically with development. As reported earlier, we have attempted, unsuccessfully to examine Wnt expression with antibodies. We therefore decided to approach their potential roles genetically.

Wnts-4, 5a, 5b, 6, 7b, 10a and 10b are expressed in either the embryonic or adult mammary gland. Although not detected in the mammary gland in the original study, Wnt-7a is expressed in mammary epithelial cell lines.Our laboratory has generated mutants in Wnt-4,5a,5b,7a,7b, in addition Wnt-6 has been generated by Dr. Andreas Kispert, part of its analysis is in collaboration with my group, and Dr. Philip Leder has reported on Wnt-10b mutants (we originally targeted this allele as part of this proposal but our single clone did not go through the germ line and we discontinued this approach on hearing of Dr. Leder's work). Of these only Wnt-5b, Wnt-6, Wnt-7a and Wnt-10b are viable. A mild phenotype was reported for Wnt-10b [P. Leder, personal communication]. However, Wnt-5b and Wnt-6, Wnt-7a mutants have no obvious phenotypes. We reported previously preliminary studies that suggested a slight delay in end bud growth in Wnt-7a mutants, but this was not substantiated by further analysis.

Of the other Wnts, mutations in Wnt-4, Wnt-5a and Wnt-7b are recessive lethals. Wnt-7b at 9.5 dpc, the other two at birth. Clearly this presents an obstacle to examining their roles in the adult , one which we attempted to overcome in collaboration with Dr. Cathrin Briskin in Dr. Robert Weinberg's laboratory at the Whitehead Institute. Dr. Brisken transplanted the mammary epithelium at birth into cleared fat pads of wild type weanlings. These experiments are difficult and have a considerable delay. It is largely as a result of the time needed to try and bring these studies to a satisfactory conclusion that we applied for a no-cost extension.

Mammary gland anlagen were isolated at 14.5 dpc and grafted into the cleared fat pad of syngeneic hosts. Analysis of graft and contralateral host tissue was performed in virgin, pregnant or lactating mice. We observed a decrease in ductal complexity which was specific to mid-pregnancy stages of mammary development (see Appendix, Figure 1). A similar phenotype has been reported for mutants in the progesterone receptor, also analysed by Dr. Brisken and Weinberg [58], and the initial findings suggest that Wnt-4, but not other Wnts, is upregulated following progesterone treatment (Appendix, Figure 2) suggesting that Wnt-4 may be a local mediator of progesterone action. Wnt-4 appears to be down-regulated in progesterone receptor mutants (Appendix, Figure 3).This work is being prepared for publication and a preprint of a current draft is enclosed. In addition, grafts of mammary gland tissue from Wnt-5a mutants were performed but await analysis.

The intrinsic problems in trying to analyze the adult mammary phenotype of a gene required for embryonic development, a problem not unique to ourselves but one shared by the community lead us to develop some novel transgenic strains. We placed the P1 phage integrase, CRE, under the control of a keratin 14 promoter. K14 is expressed in the ectoderm of the skin, and in its derivatives such as the early involuting mammary gland. Seven founder lines were obtained and two of these were tested for CRE activity by mating to a reporter strain which has a silent lacZ allele knocked into the ubiquitously expressed Rosa26 locus, requiring CRE mediated recombination for activation [59]. Figure 4 (see Appendix) illustrates that CRE activates the reporter throughout the mammary gland epithelium. Thus, this strain should allow the complete removal of gene function form the mammary epithelium. A second construct which encodes a tamoxifen (TM) inducible form of CRE recombinase for temporal control of gene targeting was

also injected. We obtained 21 founders and have not determined whether we are able to activate CRE by TM but will make any useful strains available. Finally, in terms of general reagents we generated a K14 GAL4 construct for bigenic transactivation of gene expression in the mammary gland. We obtained 10 lines, examined the activity of three and none gave transactivation of reporter gene expression. It is not clear why as we have used GAL4 mediated transactivation in CNS analyses and it has been shown to work in mammary epithelium [60].

At present there is a relatively poor understanding of the transcriptional targets of Wnt-signaling. One likely target from the study of Wnts in the limb is Lmx-1b, a transcriptional regulator. In the limb, Wnt-7a is a dorsalizing signal supplied by the dorsal ectoderm operating on a downstream mesenchymal target, a transcriptional regulator, Lmx-1b [61]. We have examined Lmx-1b in the mammary gland, and discovered that it is present from the initiation of mammary development at 11.5 dpc (Fig. 3). In conjunction with our colleague Dr. Randy Johnson at M. D. Anderson, in Texas, we have looked at mammary gland in Lmx-1b mutants generated in his group [62]. Interestingly, they lack any detectable mammary gland at 18.5 dpc (Appendix, Figure 5). One interpretation is that Lmx-1b may be downstream of other Wnt-signals in the mammary gland. Moreover, our initial analysis suggested that Lmx-1b was only expressed in the mammary gland bud, and not in other ectodermal placode suggesting that Lmx-1b may give mammary specificity to cell differentiation or branching. We tested this idea by generating a number of K14-Lmx-1b transgenic lines (eleven). In these, Lmx-1b would be predicted to be ectopically expressed in hair follicles and teeth, possibly resulting in the ectopic development of mammary structures in their place. However, despite the fact that the transgene was expressed as expected, and active (as evidenced by an eye phenotype which was not followed up), we saw no evidence for ectopic mammary gland development. In the course of analyzing these mice we determined that whereas Lmx-1b was expressed at high levels in the mammary gland, it was also expressed at low levels in wild-type hair follicles. Consequently, our original hypothesis was not likely to be correct. We also attempted to determine whether Lmx-1b might be a target of Wnt-10b signaling. However, the K14 GAL4/UAS Wnt-10b bigenic approach did not result in transactivation.(see earlier) due to some problem in the GAL4 transgene which was not expressed.

Recommendation for future studies

Mouse genetics offers a powerful approach toward analysis of gene function. However, the late development of the mammary gland and need to study gene function at different stages of mammary gland development suggest that unique genetic tools should be developed to facilitate genetic dissection of mammary gland development. We report here a strain of mouse which should allow CRE mediated recombination throughout the entire mammary gland epithelium from its earliest stages. Recombination can be used to turn gene activity on or off. K14 restricts its activity to a small subset of tissues in the embryo and adult, which will most likely overcome viability issues for many genes. There is a clear need for other such tools and this might reasonably be a subject for discussion. The problem of dissecting Wnt-function and the difficulties we have encountered is, I am sure, just one illustration of the problem.

4) **Wnt receptors**

Frizzled family members have been identified as Wnt-receptors over the course of this grant [51]. Although this is almost certainly the case, the specific designation of individual Frizzleds (Fz) as receptors for specific Wnts has not been straightforward. In vitro studies have demonstrated broad specficity. We identified one receptor, Fz-6, in the mammary gland. Our original plan was to address its function by transgenic approaches. However, on reviewing the generally poor information on Fz expression in the mammary gland, coupled with the poor understanding of which Wnts are functional we discontinued this effort in favor of the transgenic studies above.

Recommendation for future studies

Understanding Wnt function will require a thorough understanding of how relevant signals are received and transduced in the mammary gland. Unfortunately, this is likely to be a big task and can only be interpreted when all potential players are identified, their functions dissected genetically, and this information confirmed biochemically.The likely importance of this family suggests that this is a worthy goal.

5) Human *Wnt*-clones

We proposed to identify a number of human Wnt clones and distribute these for clinical studies. Clones have been dispersed and have been used in a number of studies with some evidence of amplification in human breast tumors[52].

Recommendation for future studies

Given that the likelihood that all expressed sequences for the human genome will have been identified in the next two years, it seems reasonable to wait until this time to collect together the full panoply of Wnt signaling reagents. This should be tremendously useful to the medical community.

7. KEY RESEARCH ACCOMPLISHMENTS

- characterized Wnt-expression in mammary gland
- generated Wnt5b mutants (no mammary phenotype)
- demonstrated role for Wnt4 in ductal branching
- linked Wnt4 to progesterone pathway
- produced mouse strain (K14CRE) for mammary epithelial gene modification in embryo
- produced mouse strains (K14CRE-ER™) for potential drug (tamoxifen) inducible gene modification in mammary epithelium
- demonstrated requirement for Lmx1b in mammary gland development
- showed that Fz-6 is expressed in mammary epithelium
- distributed human Wnt clones for studies of human tumorigenesis

8. REPORTABLE OUTCOMES

- manuscripts (see bibliography)
- mouse strains (Wnt5b mutant, K14CRE, K14CRE-ER™

9. CONCLUSIONS

These studies have started to address the normal roles of Wnt-signals and their possible targets in the developing mammary gland. Unpublished work from others has shown requirement for Wnt-10b [P. Leder, personal communication], our own work indicates that Wnt-4 is required for ductal branching during pregnancy and suggest a possible link with progesterone signaling. However, our analyses, and a consideration of future work on Wnt and other signaling pathways indicates that new tools must be developed to functionally dissect the genetic regulation of this organ. As a small step we have generated a strain of mouse which should be helpful in recombination mediated activation or removal of gene function from the mammary gland, with reasonable specificty.

8. REFERENCES

1. Kratochwil, K. (1987). Epithelium - Mesenchyme interaction in the fetal mammary gland. Cellular and Molecular Biology of Mammary Cancer, (Eds. Medina, D., Kidwell, W., Heppner, and Anderson, E.) *Plenum Publishing Corp.* 67-80.
2. Raynaud, A. (1961). Morphogenesis of the mammary gland. Milk: the mammary glands and its secretions. (Eds. Kon, S.K. and Cowie, A.T.) Academic Press, *1,* 3-46.
3. Balinsky, B.I. (1950). On the prenatal growth of the mammary gland rudiment in the mouse. Journal of Anatomy, *84*, 227-235.

4. Sakakura, T. (1987). Mammary embryogenesis. The Mammary Glands, Development, Regulation and Function. (Eds. Neville, D.D. and Daniel, C.W.) *Plenum Publishing Corp, New York,* 37-66.
5. Propper, A. (1968). Relations epidermo-mesodermiques dans la differenication de l'ebauche mammaire de embryon de lapin. Ann. Embryol. Morphog. *1*, 151-160.
6. Kartochwil, K., and Schwartz, P. (1976). Tissue interaction in androgen response of embryonic mammary rudiment of mouse: identification of target tissue for testosterone. Proc. Nat. Acad. Sci. USA, *73*, 4041-4044.
7. Wasner, G., Hennermann, I., and Kratochwil, K. (1983). Ontogeny of mesenchymal androgen receptors in the embryonic mouse mammary gland. Endocrinology *113*, 1171-1780.
8. Durnberger, H. and Kratochwil, K. (1980). Specificity of tissue interaction and origin of mesenchymal cells in the androgen response of the embryonic mammary gland. Cell, *19*, 465-471.
9. Sakakura, T., Sakagami, Y., and Nishizuka, Y. (1982). Dual origin of mesenchymal tissues participating in mouse mammary gland embryogenesis. Dev. Biol., *91*, 202-207.
10. Kratochwil, K. (1969). Organ specificity in mesenchymal induction demonstrated in the embryonic development of the mammary gland of the mouse. Dev. Biol, *20*, 46-71.
11. Borellini, F. and Oka, T. (1989). Growth control and differentiation in mammary epithelial cells. Environmental Health Perspectives, *80*, 85-99.
12. Daniel, C.W. and Silberstein, G.B. (1987). Postnatal development of the rodent mammary gland. The Mammary Glands, Development, Regulation and Function (Eds. Neville, D.D. and Daniel, C.W.) *Plenum Publishing Corp. New York,* 3-36.
13. Ceriani, R.I. (1970). Fetal mammary gland differentation *in vitro* in response to hormones. II. Biochemical Findings, Dev. Biol. *21*, 530-546.
14. Coleman, S. Silberstein, G.B. and Daniel, C.W. (1988). Ductal morphogenesis in the mouse mammary gland: evidence supporting a role for epidermal growth factor. Dev. Biol., *127*, 304-315.
15. Coleman, S. and Daniel, C.W. (1990). Inhibition of mouse mammary ductal morphogenesis and down-regulaton of the EGF receptor by epidermal growth factor. Deve. Biol., *137*, 425-433.
16. Silberstein, G.B. and Daniel, C.W. (1987b). Reversible inhibition of mammary gland growth by transforming growth factor-beta. Science, *237*, 291-293.
17. Daniel, C.W., Silberstein, G.B., Van Horn, K., Strickland, P. and Robinson, S. (1989). TGF-β1-induced inhibition of mouse mammary ductal growth: developmental specificity and characterization. Deve. Biol., *135*, 20-30.
18. Vonderhaar, B.K. (1987). Local effects of EGF, α-TGF, and EGF-like growth factors on lobuloalveolar development of the mouse mammary gland *in vivo*. J. Cell. Physiol. *132*, 581-584.
19. Sandgren, E. P., Luetteka, N.C., Palmiter, R.D., Brinster, R.L. and Lee, D.C. (1990). Overexpression of TGFα in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. Cell, *61*, 1121-1135.
20. Jhappan, C. Stahle, C., Harkins, R.N. Fausto, N. Smith, G.H., Merlino, G.T. (1990). TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell, *61*, 1137-1146.
21. Matsui, Y., Halter, S.A., Holt, J.T., Hogan, B.L.M., and Coffey, R.J. (1990). Development of mammary hyperplasia and neoplasia in MMTV-TGFα transgenic mice. Cell, *61*, 1147-1155.
22. Nusse, R. (1988a). The activation of cellular oncogenes by proviral insertion in murine mammary tumors. In M. E. Lippman & R. Dickson (Eds.), Breast Cancer: Cellular and Molecular Biology, Boston: *Kluwer Academic Publishers*, 283-306
23. Nusse, R., Van Ooyen, A., Cox, D., Fung, Y.-K. T., & Varmus, H. E. (1984). Mode of proviral activation of a putative mammary oncogene (*int*-1) on mouse chromosome 15. Nature (London), *307*, 131-136.
24. Roelink, H., Wagenaar, E., Lopes da Silva, S., & Nusse, R. (1990). *wnt*-3, a gene activated by proviral insertion in mouse mammary tumors, is homologous to *int*-1/*Wnt*-1 and is normally expressed in mouse embryos and adult brain. Proc. Natl. Acad. Sci. USA, *87*, 4519-4523.

25. Dickson, C., Smith, R., Brookes, S., and Peters, G. (1984). Tumorigenesis by mouse mammary tumor virus: proviral activation of a cellular gene in the common integration region *int*-2. Cell, *37,* 529-536.
26. Peters, G., Brookes, S., Smith, R., Placzek, M., and Dickson, C. (1989). The mouse homolog of the *hst/k-FGF* gene is adjacent to *int-2* and is activated by proviral insertion in some virally induced mammary tumors. Proc. Natl. Acad. Sci., USA., *86,* 5678-5682.
27. Peters, G., Lee, A.E., Dickson, C., (1986). Concerted activation of two potential proto-oncogenes in carcinomas induced by mouse mammary tumor virus. Nature, *320,* 628-631.
28. Brown, A. M. C., Wildin, R. S., Prendergast, T. J., & Varmus, H. E. (1986). A retrovirus expression vector expressing the putative mammary oncogene *int*-1 causes partial transformation of a mammary epithelial cell line. Cell, *46,* 1001-1009.
29. Jue, S. F., Bradley, R. S., Rudnicki, J. A., Varmus, H. E., & Brown, A. M. C. (1992). The mouse *Wnt*-1 gene can act via a paracrine mechanism in transformation of mammary epithelial cells. Mol. Cell. Biol., *12,* 321-328.
30. Rijsewijk, F., van Deemter, L., Wagenaar, E., Sonnenberg, A., & Nüsse, R. (1987a). Transfection of the *int*-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumoriginicity. EMBO J., *6,* 127-131.
31. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T., & Varmus, H. E. (1988). Expression of the *int*-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell, *55,* 619-625.
32. Kwan, H., Pecenka, V., Tsukamoto, A., Parslow, T.G., Guzman, R., Lin, T-P., Muller, W.J., Lee, F.S., Leder, P. and Varmus, H.E. (1992). Transgenes expressing the*Wnt-1* and *int-2* proto-oncogenes cooperate during mammary carcinogenesis in Doubly transgenic mice. Mol. Cell. Biol., *12,* 147-154.
33. Wainwright, B. J., Scambler, P. J., Stanier, P., Watson, E. K., Bell, G., Wicking, C., Estivill, X., Courtney, M., Bowe, A., Pedersen, P. S., Williamson, R., & Farrall, M. (1988). Isolation of a human gene with protein sequence similarity to human and murine *int*-1 and the *Drosophila* segment polarity gene *wingless*. EMBO J., *7,* 1743-1748.
34. McMahon, J. A., & McMahon, A. P. (1989c). Nucleotide sequence, chromosomal localization and developmental expression of the mouse *int*-1-related gene. Development, *107,* 643-651.
35. Roelink, H., Wagenaar, E., and Nusse, R. (1992). Amplification and proviral activation of several *Wnt* genes during progression and clonal variation of mouse mammary tumors. Oncogene, *7*(3), 487-492.
36. Roelink, H., & Nusse, R. (1991). Expression of two members of the *Wnt* family during mouse development - restricted temporal and spatial patterns in the developing neural tube. Genes & Dev., *5,* 381-388.
37. Gavin, B., McMahon, J. A., & McMahon, A. P. (1990). Expression of multiple novel *Wnt*-1/*int*-1-related genes during fetal and adult mouse development. Genes & Dev., *4,* 2319-2332.
38. Brown, A. M. C., Papkoff, J., Fung, Y.-K. T., Shackleford, G. M., & Varmus, H. E. (1987). Identification of protein products encoded by proto-oncogene *int*-1. Mol. Cell. Biol., *7,* 3971-3977.
39. Papkoff, J. A., Brown, A. M. C., & Varmus, H. E. (1987). The *int*-1 proto-oncogene products are glycoproteins that appear to enter the secretory pathway. Mol. Cell. Biol., *7,* 3978-3984.
40. Papkoff, J. (1989). Inducible overexpression and secretion of *int*-1 protein. Mol. Cell. Biol., *9,* 3377-3384.
41. Papkoff, J., & Schryver, B. (1990). Secreted *int*-1 protein is associated with the cell surface. Mol. Cell. Biol., *10,* 2723-2730.
42. Bradley, R. S., & Brown, A. M. C. (1990). The proto-oncogene *int*-1 encodes a secreted protein associated with the extracellular matrix. EMBO. J., *9,* 1569-1575.
43. Mason, J. O., Kitajewski, J., & Varmus, H. E. (1992). Mutational analysis of mouse *Wnt*-1 identifies two temperature-sensitive alleles and attributes of *Wnt*-1 protein essential for transformation of a mammary cell line. Mol. Cell. Biol.
44. Blasband, A., Schryver, B., & Papkoff, J. (1992). The biochemical properties and transforming potential of human *Wnt*-2 are similar to *Wnt*-1. Oncogene, *7,* 153-161.
45. McMahon, A. P. (1992). The Wnt family of developmental regulators. Trends in Genetics, *8,* 236-242.
46. Nusse, R., & Varmus, H. E. (1992). Wnt-genes. Cell, *69,* 1073-1087.

47. Gavin, B. J., & McMahon, A. P. (1992). Differential regulation of the *wnt*-gene family during pregnancy and lactation suggests a role in post natal development of the mammary gland. Molec. Cell Biol., *12*(5), 2418 - 2423.
47b. Bühler, T.A., et.al. (1993). Localization and quantification of Wnt-2 gene expression in mouse mammary development. Dev. Biol. *155,* 87-96.
48. Wong, G.T., Gavin, B.J. and McMahon, A.P. (1994). Differential transformation of mammary epithelial cells by *Wnt* genes. Mol. Cell. Biol., *14*, 6278-6286.
49. Edwards, P.A.W., Hiby, S.E., Papkoff, J. and Bradbury, J.M. (1992). Hyperplasia of mouse mammary epithelium induced by expression of the *Wnt-1 (int-1)* oncogene in reconstituted mammary gland. Oncogene, *7,* 2041-2051.
50. Hanks, M., Wurst, W., Anson-CartwrightL., Auerbach, A. B. and Joyner, A. (1995). Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. Science 269, 679-682.
51. Bhanot, P. et al. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature 382, 225-230.
52. Huguet, E.L., McMahon, J.A. McMahon, A.P. and Harris, A.L. (1994). Differential expression of human wnt genes 2,3,4 and 7b in human breast cell lines and normal and diseased states of human breast tissue. Cancer Research *54*, 2615-21.
53. Lee, F. S. et al. (1995). Insertional mutagensis identifies a member of the Wnt gene family as a candidate oncogene in the mammary epithelium of int-2/Fgf-3 transgenic mice. PNAS 92, 2268-2272.
54. Genderen, V. c., Okamura, R.M., Farinas, I., Quo, R.G., Parslow, T.G., Bruhn, L. and Grosschedl, R. (1994). Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1 deficient mice. Genes Dev., 8, 691-703.
55. Testsu, O. and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398, 422-426.
56. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/Lef-1 pathway. Proc Natl. Acad. Sci. 96, 5522-5527.
57. Bejosovec, A. and Wieschaus, E. (1995). Signaling activities of the Drosophila wingless gene are separately mutable and appear to be transduced at the cell surface. Genetics, 139, 309-320.
58. Brisken, C., Kaur, S., Chavarria, TE, Binart, N., Sutherland, RL., Weinberg, RA, Kelley, PA, Ormandy, CJ. (1999). Prolactin controls mammary gland development via direct and indirect mechanisms. Dev. Biol. 210, 96-106.
59. Soriano, P. (1999). Generalized lacZ expression with the Rosa26 cre reporter strain. Nat. Genet. 21, 70-71.
60. Orntiz, D., Moreadith, R.W. and Leder, P. (1991). Binary system for regulating transgene expression in mice: targeting int-2 gene expression with yeast GAL4/UAS control elements. Proc Natl. Acad. Sci 88, 698-702.
61. Riddle, R.D., Ensini, M., Nelson, C., Tsuchida, T., Jessell, TM and Tabin, C. (1995). Induction of the LIM homeobox domain gene Lmx1 by Wnt-7a establishes dorsoventral polarity in the vertebrate limb. Cell 83, 631-640.
62. Chen, H., Lun, Y., Ovchinnikov, D., Kokubo, H., Oberg, K.C, Pepicelli, C.V., Gan, L., Lee. B., and Johnson, R. L. (1998). Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. Nat. Genet. 19, 51-55.

9. Appendix

Figure 1. Comparison of epithelial branching in mammary gland grafts from Wnt4 mutants (-/-) and wild-type (+/+).
Figure 2. RT-PCR analysis of Wnt gene expression in the mammary glands prior too, and 8 hours after progesterone treatment. Wnt-4 shows up-regulation, not observed for other wnts and controls (K18 and GAPDH).
Figure 3. RT-PCR analysis indicates downregulation of Wnt-4 in progesterone receptor mutant. Lanes represent a dilution series.
Figure 4. K14CRE mediated activation of a ROSA26 lacZ allele in the developing mammary gland at birth.
Figure 5. Lmx1b is required for embryonic development of the mammary gland.

11) Bibliography .

Burrus, L. W. and McMahon, A.P. (1995). Biochemical analysis of murine Wnt proteins reveals both shared and distinct properties. Exp. Cell. Res. 220, 363-373.

Van Bokhaven, H., Kissing, J., Schepens, M., van Beersum, S., Simons, A., Riegman, P., McMahon, J.A., McMahon, A.P. and Brunner, H.G. (1997). Assignment of Wnt-7b to human chromosome band 22q13 by in situ hybridization. Cytogenet. Cell Genet., 77, 288-289.

Brisken, C., Weinberg R. and McMahon, A. P. (1999). Evidence for an interaction between PR and Wnt-4 in the mammary gland. In preparation.for Nature, draft supplied.

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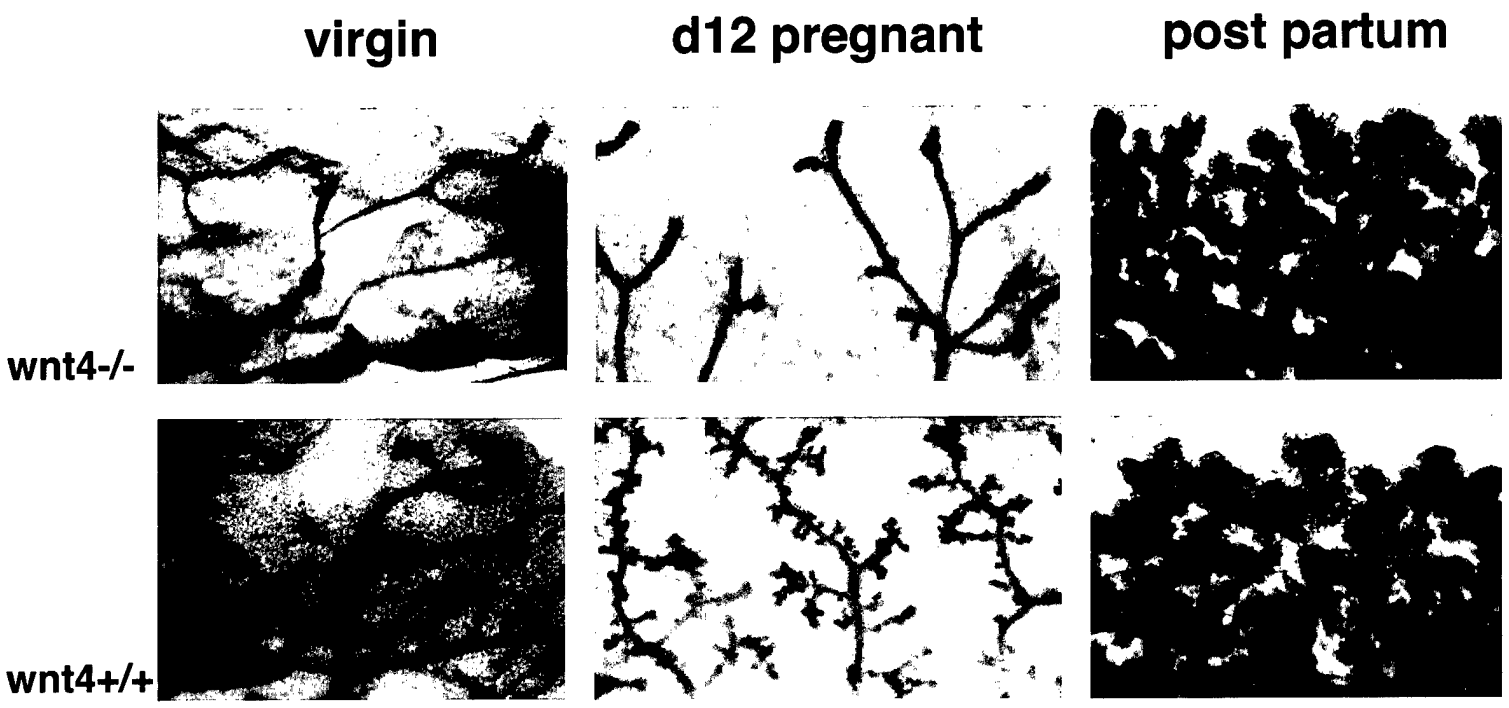

Figure 1

Primary mouse mammary epithelial cells:
8 hr treatment with progesterone (R5020)

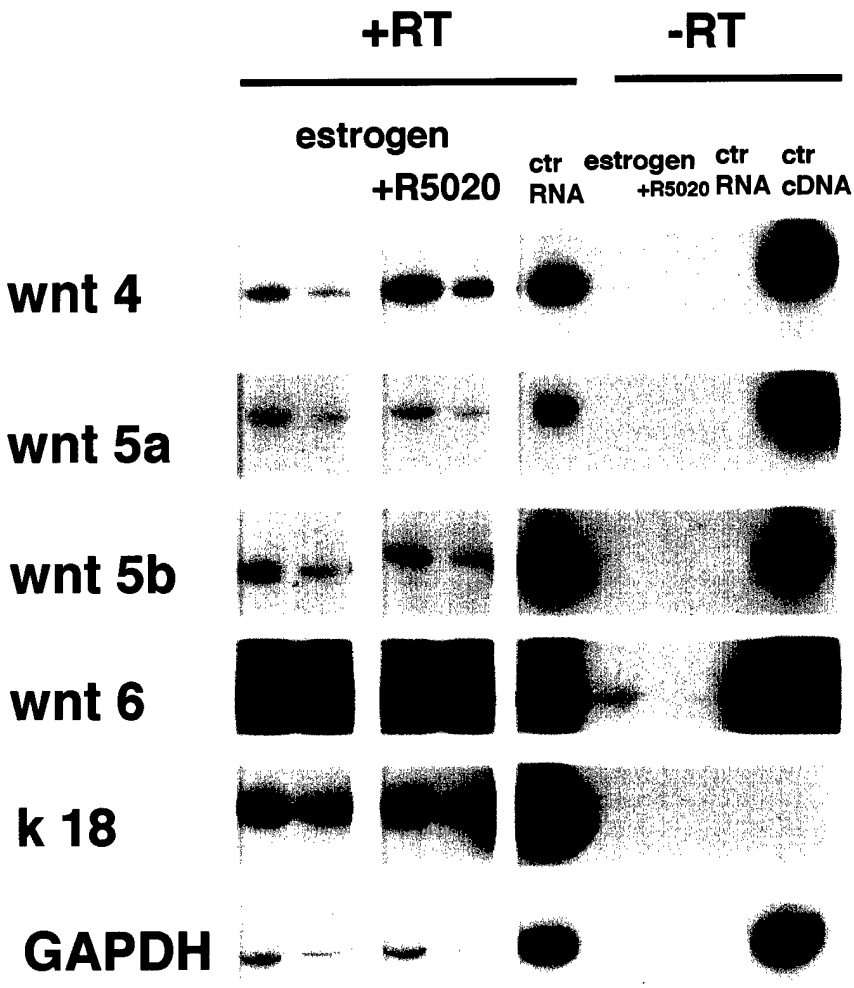

Figure 2

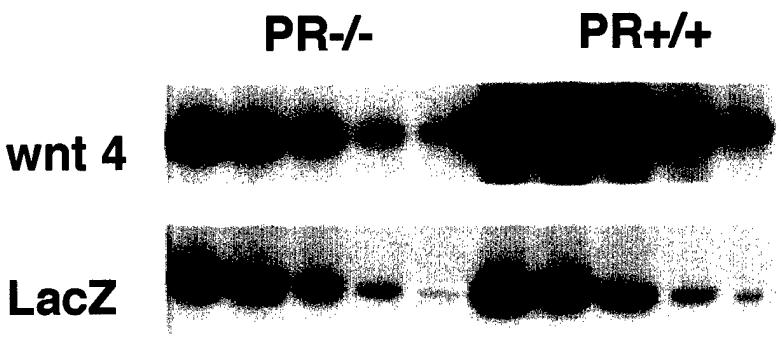
RT-PCR: engrafted mammary glands,
recipient day 12.5 pregnant
PR-/-
PR+/+
wnt 4
LacZ

Figure 3

K14Cre+/-, Rosa26c/+

WT

E19.5

Figure 4

Lmx1b Regulates Mammary Gland Development

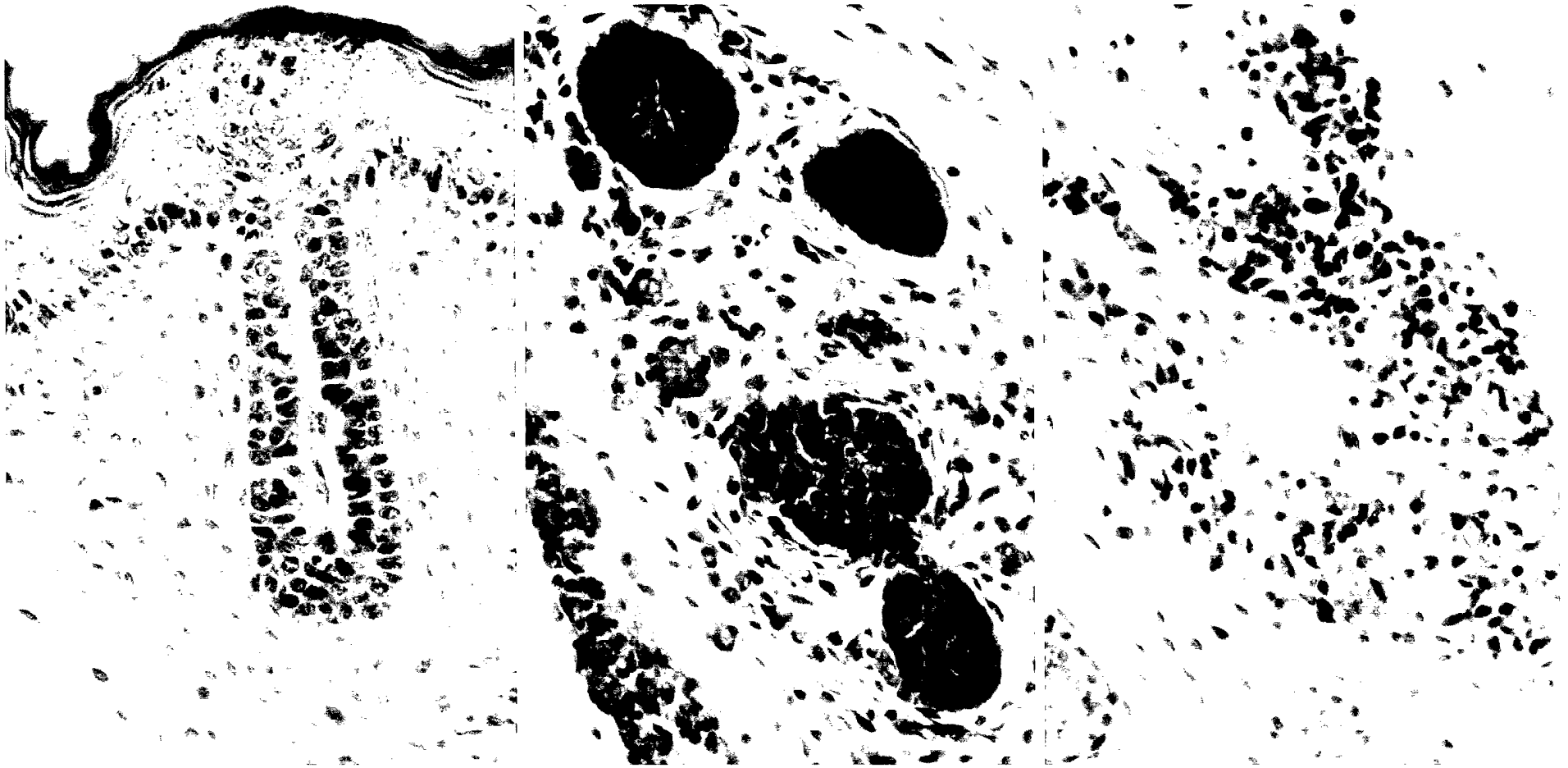

Figure 5

Wnt4 Plays an Essential Role in Mammary Gland Sidebranching, Downstream of Progesterone Signaling

Cathrin Brisken, Anna Heineman, Tony Chavarria, Brian Elenbaas, Klaus Kratochwil, Andrew McMahon and Robert A. Weinberg

The milk ducts branch extensively under the influence of the female reproductive hormones[1]. This arborization fails to occur in the mammary epithelium of mice lacking the progesterone receptor (PR)[2,3]. We now find that this defect can be overcome by ectopic expression of the mammary protooncogene Wnt1 and that Wnt1 acts in a paracrine fashion to elicit this response. The related Wnt4 protein, which acts similarly to Wnt1[4], is normally expressed in the mammary epithelium at a time when sidebranching occurs[5,6]. We rescue mammary epithelium from $Wnt4^{-/-}$ mice which die at birth[7] by transplantation and show that $wnt4^{-/-}$ epithelium fails to undergo normal sidebranching early in pregnancy. These observations indicate that Wnt4 acts as a downstream effector of progesterone during this period, and indeed we find that progesterone can induce an increase in Wnt4 expression, suggesting that progesterone acts as a morphogen in the breast through its ability to induce Wnt4 synthesis in mammary epithelial cells.

Development of the mammary gland occurs largely postnatally under the control of the female reproductive hormones estrogen, progesterone and prolactin[1]. The mechanisms that enable these systemic factors to control locally acting morphogens remain largely unknown. Recently, we have shown that progesterone acts via the PR in the mammary epithelium to induce sidebranching by a paracrine mechanism[2].

We speculated that wnts or FGFs, two classes of paracrine factors, might be downstream morphogenetic effectors of progesterone in the process of sidebranching. Wnts play important roles in the development of various vertebrate and invertebrate tissues[8,9]. These factors are secreted glycoproteins that bind to members of the Frizzled family of seven-transmembrane-domain receptors. Several *wnt* genes function as oncogenes in the mouse breast when activated by insertion of the provirus mouse mammary tumor virus (MMTV)[10-12] or when overexpressed[13]. Here,

we identify wnt4 as a target of progesterone and assign a physiological function to wnt4 in ductal sidebranching.

To test whether a Wnt factor might function downstream of progesterone signaling in triggering ductal sidebranching in the breast, we crossed mice carrying an MMTV LTR-driven *Wnt-1* transgene[13] (kind gift H. Varmus) with $PR^{-/+}$ mice, to generate *Wnt-1* transgenic females that were either wt or homozygous mutant at the PR locus. We then sought to test whether the ectopically expressed Wnt1 protein might restore the sidebranching that is lacking in $PR^{-/-}$ mammary ducts.

Since the deletion of the PR affects the reproductive system of female mice[14], any differences that we might have observed between the mammary glands of $PR^{-/-}$wnt1 and $PR^{+/+}$ wnt1 mice could have been attributed to systemic endocrinal disturbances rather than to localized morphogenetic defects. To eliminate this concern, mammary epithelia were removed from mice of both genotypes and transplanted into 3-week-old $PR^{+/+}$ females whose inguinal mammary glands had been surgically cleared of endogenous epithelium. When epithelial tissue[15] or primary cells[16] are engrafted into such cleared fat pads, they are able to form a new ductal sytem. $RAG1^{-/-}$ females were used as recipients of these grafts, as mice of this genotype are immunocompromised and therefore able to accept allografts[2,17].

Ten weeks after grafting, the recipients' endogenous breasts showed a simple ductal system characteristic of a 13-week-old virgin mouse. However, both implants, MMTV *wnt-1* .PR wt and MMTV *wnt-1* .$PR^{-/-}$, showed increased branching (fig1). Thus, overexpression of wnt1 can induce sidebranching in a $PR^{-/-}$ epithelium in which sidebranching is otherwise not observeable, suggesting that wnt signaling may act downstream of progesterone signaling.

We reported previously that in chimeric epithelia derived from mixed wt and $PR^{-/-}$ MECs, the branching defect of the mutant MECs could be rescued if these cells grew in close proximity to their wt counterparts[2]. This suggested that progesterone elicits its morphogenetic effects, at least in part, by causing PR-positive MECs to release a factor that acts over short distances on other cells within the breast.

To test whether the wnt-1 produced by the MMTV *wnt-1* MECs also acts in a paracrine fashion to induce sidebranching, we mixed these cells with MECs derived from ROSA26 mice[18], which carry a ubiquitously expressed LacZ transgene which makes their identification possible upon wholemount

analysis of breast tissue. As expected, the MMTV *wnt-1* transgenic cells, stained in red, showed increased sidebranching (Fig.2). In addition, the blue wt cells carrying the lacZ transgene and located adjacent to these MMTV-int1 MECs also showed increased sidebranching. This indicates that secreted wnt1 is sufficient to cause sidebranching and that wnt1, like the factor released by PR-positive cells, acts in a paracrine fashion to induce sidebranching.

While these experiments indicated that a Wnt protein was sufficient for sidebranching, being able to mimic the effects normally elicited by progesterone, they did not resolve whether a Wnt factor plays an essential role in the normal process. Wnt1 itself is not normally expressed in the mammary gland, but the related wnt4 is expressed when sidebranching occurs during early/mid pregnancy[5,6]. To evaluate the specific role played by Wnt4 in mammary morphogenesis, we resorted to mice lacking both copies of the *wnt-4* gene[7]. These mice die perinatally due to kidney failure[7], precluding analysis of subsequent mammary development. We rescued the mammary buds from 14.5 day-old $Wnt4^{-/-}$ and wt embryos by implanting them into the cleared fat pads of wild type hosts. Both types of implants gave rise to normal ductal systems in virgin recipients (fig. 3, left panel). However, in a series of 10 recipients analyzed around day 12 of pregnancy, $wnt4^{-/-}$ implants showed consistently less ductal complexity than their wt counterparts (fig. 3, central panel). This defect did not endure, by the end of pregnancy, both implants showed full ductal arborization (fig. 3, right panel).

Taken together, these data indicate that wnt4 is required for sidebranching during mid-pregnancy. However, during later pregnancy, other factors, including possibly Wnt5b and Wnt6 upregulated at that time[5,6], are able to compensate for the early absence of Wnt4 and restore full mammary ductal morphology.

Since sidebranching is normally controlled by progesterone, we proposed that during early and mid-pregnancy, Wnt4 is an essential effector of the progesterone-triggered sidebranching program. To test whether wnt4 expression is controlled by progesterone, we injected ovariectomized mice, which lack both progesterone and estrogen, with either estrogen alone, or estrogen plus progesterone, or vehicle alone for 20 days as described[19]. Estrogen was injected to induce, via the estrogen receptor, expression of the PR in MECs[x]. We presumed that in animals that also received progesterone, it would be able to activate the estrogen-induced PR. At the end of this treatment, one mammary gland was analyzed by wholemount microscopy to

adequate gonadectomy and hormone introduction. RNA was extracted from the remaining breasts and assayed by RT-PCR for levels of GAPDH and wnt4 RNA expression at ranges of serial dilutions that ensured a linear signal response to RNA levels. We observed a slight increase in the expression of wnt4 RNA in response to estrogen treatment alone, and a five-fold stronger increase progesterone plus estrogen treatment (Fig. 4). Thus, consistent with wnt4 acting downstream of the PR, the administration of progesterone leads to the induction of wnt4 expression in vivo.

We wished to confirm that the observed Wnt4 induction was dependent locally upon the presence of the PR. To do so, we assayed wnt4 expression in pregnant mice that had been engrafted with $PR^{-/-}$ MECs on one side and $PR^{-/-}$ MECs PRwt epithelium contraterally. The transplanted epithelial cells also carried a LacZ gene and RT-PCR for LacZ was used to normalize for levels of wnt4 in the transplanted tissue. At day 12 of pregnancy the levels of LacZ are comparable between the $PR^{-/-}$ implant and its wt counterpart but we observed 3-fold difference between the levels of wnt4 in the two grafts (fig. 5). This indicated indicates that the PR is required within the mammary epithelium for the induction of wnt4.

To address whether the induction of wnt4 by progesterone is a direct effect of PR action, we treated primary MECs in culture with progesterone. As shown in figure 5, at eight hours wnt4 is significantly induced while wnt 5a, 5b and 6 show no increase in RNA levels. The induction can already be detected at 4 hours (data not shown) suggesting that the effect occurs in the PR-positive target cell. *We addressed whether the induction still occurred in the presence of the protein synthesis inhibitor cyclohexamide. An increase in wnt4 message was maske by increased basal levels of the message.*

Our findings indicate that progesterone, acting via the PR, induces Wnt4 synthesis, and that this synthesis is necessary for normal ductal sidebranching. Moreover, ectopically expressed Wnt1 can on its own elicit sidebranching, suggesting that the production of a Wnt is centrally important to progesterone-induced arborization of the mammary ductal epithelium. Wnt4 is not unique in its ability to trigger sidebranching, since later in pregnancy, the ductal epithelium of $Wnt4^{-/-}$ mice once again shows normal sidebranching. We speculate that this compensation is due to other Wnts that are expressed later in pregnancy[5,6]. While MECs ectopically expressing wnt4 give rise to sidebranches and alveolar outgrowths in virgin mice[4] we find that wnt4 is only required for sidebranching. It is conceivable that wnt4 expressed at supraphysiological levels might activate another wnt pathway

that causes alveologenesis. These observations suggest that wnts fulfil similar biochemical functions while they have different biological functions at least some of which can be explained by differences in the way they are regulated. This notion is supported by our observation that wnt4 is the only of four wnts expressed in the mammary gland during pregnancy to be induced by progesterone in primary mammary epithelial cells within 8 hours.

When the MMTV-wnt1 transgene is crossed into the estrogen receptor$^{-/-}$ background, double mutants showed increased sidebranching but the defect in ductal elongation typical of estrogen receptor$^{-/-}$ mammary glands [20,21] was not reversed. This indicates that the wnt1 effect is specific to the PR-regulated sidebranching pathway and that the two morphogenetic processes occurring during mammary gland development, estrogen-induced ductal elongation and progesterone-depended sidebranching, are mediated by different molecular mechanisms only one of which is driven by a wnt1-like substance.

Materials and Methods

Mice

ROSA26 and RAG1$^{-/-}$ mice were purchased from Jackson Laboratories. For genotyping, genomic DNA was isolated from tails and analyzed by PCR[12]. For PR PCR see (). MMTVint

Presence of the β-galactosidase transgene was tested for by subjecting a piece of tail to the X-gal stain procedure described below.

Transplantation of Mammary Epithelium

The fat pads of 3-week-old RAG1$^{-/-}$ females were cleared[X]. Pieces of mammary tissue of 1 mm diameter were removed from the nipple region of the inguinal glands from PR$^{+/+}$ and PR$^{-/-}$ females.

Cell Culture.

Embryos were harvested from wnt4$^{+/-}$ crosses on e14.5. The embryos were phenotyped based on kidney morphology. The phenotyping was subsequently confirmed by PCR-based genotyping. The mammary anlagen were dissected[X] and subsequently engrafted to cleared inguinal fat pads of three-week-old recipients.

Mammary Gland Whole Mounts

The inguinal mammary glands were dissected, spread onto a glass slide, fixed in a 1:3 mixture of glacial acetic acid: 100% ethanol, hydrated, stained overnight in 0.2% carmine (Sigma) and 0.5% $AlK(SO_4)_2$, dehydrated in graded solutions of ethanol, and cleared in BABB (benzyl alcohol and benzylbenzoate(1:2), Sigma). Pictures were taken on a Leica MZ12 stereoscope with Kodak Ektachrome 160T.

X-gal stain

The transplanted mammary glands were dissected, fixed for an hour in 4% formaldehyde in phosphate-buffered saline (PBS), washed three times over 1 hour with rinse buffer (2 mM $MgCl_2$, 0.1% sodium deoxycholate, 0.2% NP40 in PBS) and rotated in X-gal staining solution (1 mg/ml 5-Bromo-4-chloro-3-indoyl-β–D-galactopyranoside, 5 mM K-ferricyanide, 5 mM K-ferrocyanide in rinse buffer) at 37 ^{o}C for 18 hours, washed in PBS and processed for wholemounting as described above.

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References

1. Nandi, S. Endocrine Control of Mammary-Gland Development in the C3H/He Crgl Mouse. *J. Natl. Cancer Inst* **21**, 1039-1063 (1958).

2. Brisken, C. *et al.* A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proc Natl Acad Sci U S A* **95**, p5076-5081 (1998).

3. Humphreys, R., Lydon, J., O'Malley, B. & Rosen, J. Mammary gland development is mediated by both stromal and epithelial progesterone receptors. *Mol Endocrinol* **11**, p801-811 (1997).

4. Bradbury, J. M., Edwards, P. A., Niemeyer, C. C. & Dale, T. C. Wnt-4 expression induces a pregnancy-like growth pattern .

5. Gavin, B. J. & McMahon, A. P. Differential regulation of the Wnt gene family during pregnancy and lactation suggests a role in postnatal development of the mammary gland. *Mol Cell Biol* **12**, 2418-2423 (1992).
6. Weber-Hall, S. J., Phippard, D. J., Niemeyer, C. C. & Dale, T. C. Developmental and hormonal regulation of Wnt gene expression in the mouse mammary gland. *Differentiation* **57**, 205-214 (1994).
7. Stark, K., Vainio, S., Vassileva, G. & McMahon, A. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* **372**, p679-683 (1994).
8. Cadigan, K. & Nusse, R. Wnt signaling: a common theme in animal development. *Genes Dev* **11**, p3286-3305 (1997).
9. Nusse, R. & Varmus, H. Wnt genes. *Cell* **69**, p1073-1087 (1992).
10. Nusse, R. & Varmus, H. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31**, p99-109 (1982).
11. Roelink, H., Wagenaar, E., Lopes, d. S. S. & Nusse, R. Wnt-3, a gene activated by proviral insertion in mouse mammary tumors, is homologous to int-1/Wnt-1 and is normally expressed in mouse embryos and adult brain. *Proc Natl Acad Sci U S A* **87**, p4519-4523 (1990).
12. Lee, F., Lane, T., Kuo, A., Shackleford, G. & Leder, P. Insertional mutagenesis identifies a member of the Wnt gene family as a candidate oncogene in the mammary epithelium of int-2/Fgf-3 transgenic mice. *Proc Natl Acad Sci U S A* **92**, p2268-2272 (1995).
13. Tsukamoto, A., Grosschedl, R., Guzman, R., Parslow, T. & Varmus, H. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* **55**, p619-625 (1988).
14. Lydon, J. *et al.* Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev* **9**, p2266-2278 (1995).
15. DeOme, K. B., Faulkin,L. J., Jr., Bern, H. A., & Blair, P. B. Development of Mammary Tumors from Hyperplastic Alveolar Nodiles Transplanted into Gland-free Mammary fat pads of female C3H Mice. *Cancer Res* **19**, 511-520 (1959).
16. Daniel, C. W., & DeOme. *Science* **149,**, 634-636. (1965).
17. Mombaerts, P. *et al.* RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, p869-877 (1992).
18. Friedrich, G. & Soriano, P. Insertional mutagenesis by retroviruses and promoter traps in embryonic stem cells. *Methods Enzymol* **225**, 681-701.

19. Said, T., Conneely, O., Medina, D., O'Malley, B. & Lydon, J. Progesterone, in addition to estrogen, induces cyclin D1 expression in the murine mammary epithelial cell, in vivo. *Endocrinology* **138**, p3933-3939 (1997).

20. Bocchinfuso, W., Hively, W., Couse, J., Varmus, H. & Korach, K. A mouse mammary tumor virus-Wnt-1 transgene induces mammary gland hyperplasia and tumorigenesis in mice lacking estrogen receptor-alpha. *Cancer Res* **59**, p1869-1876 (1999).

21. Lubahn, D. *et al.* Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci U S A* **90**, p11162-11166 (1993).

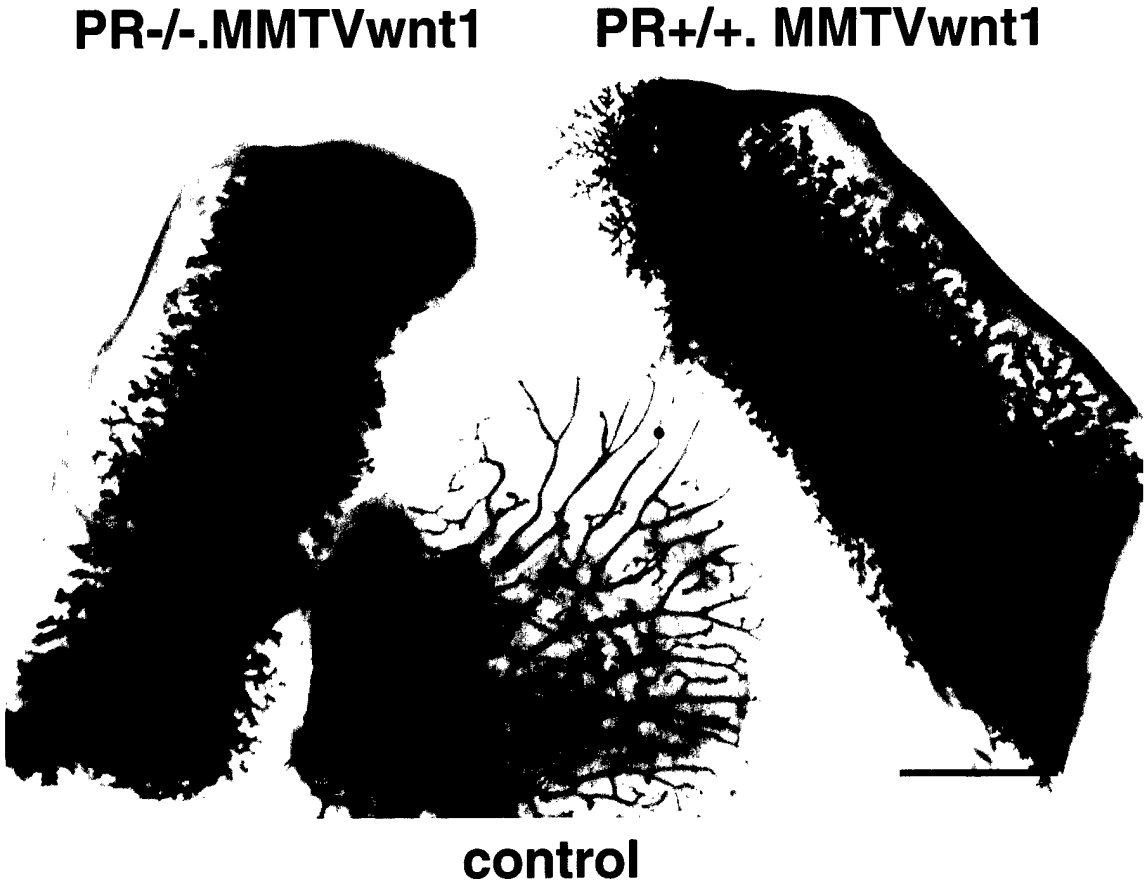


Figure 1

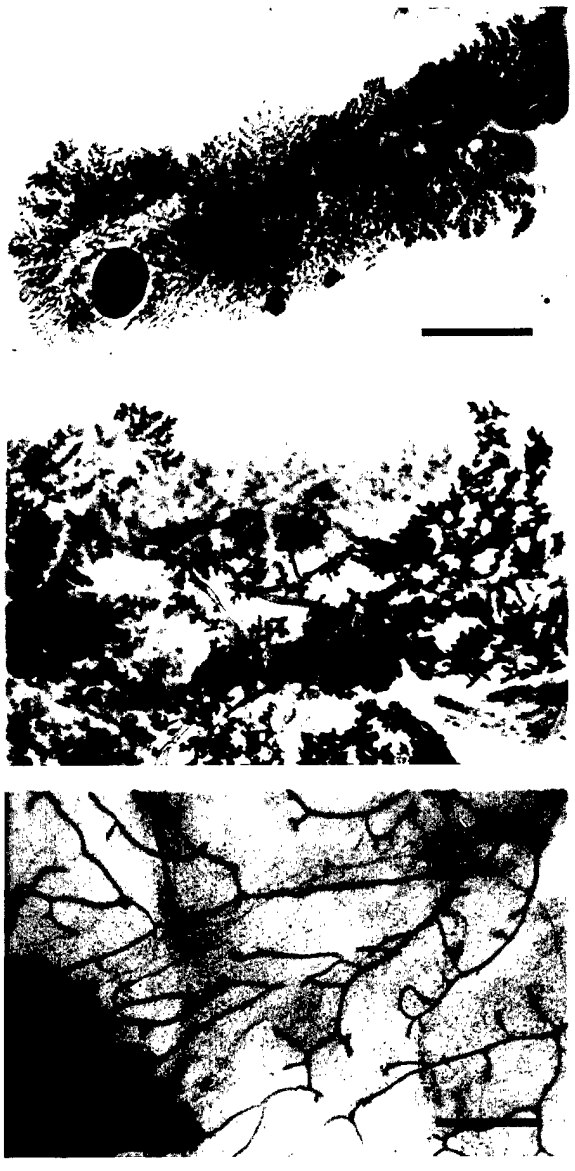
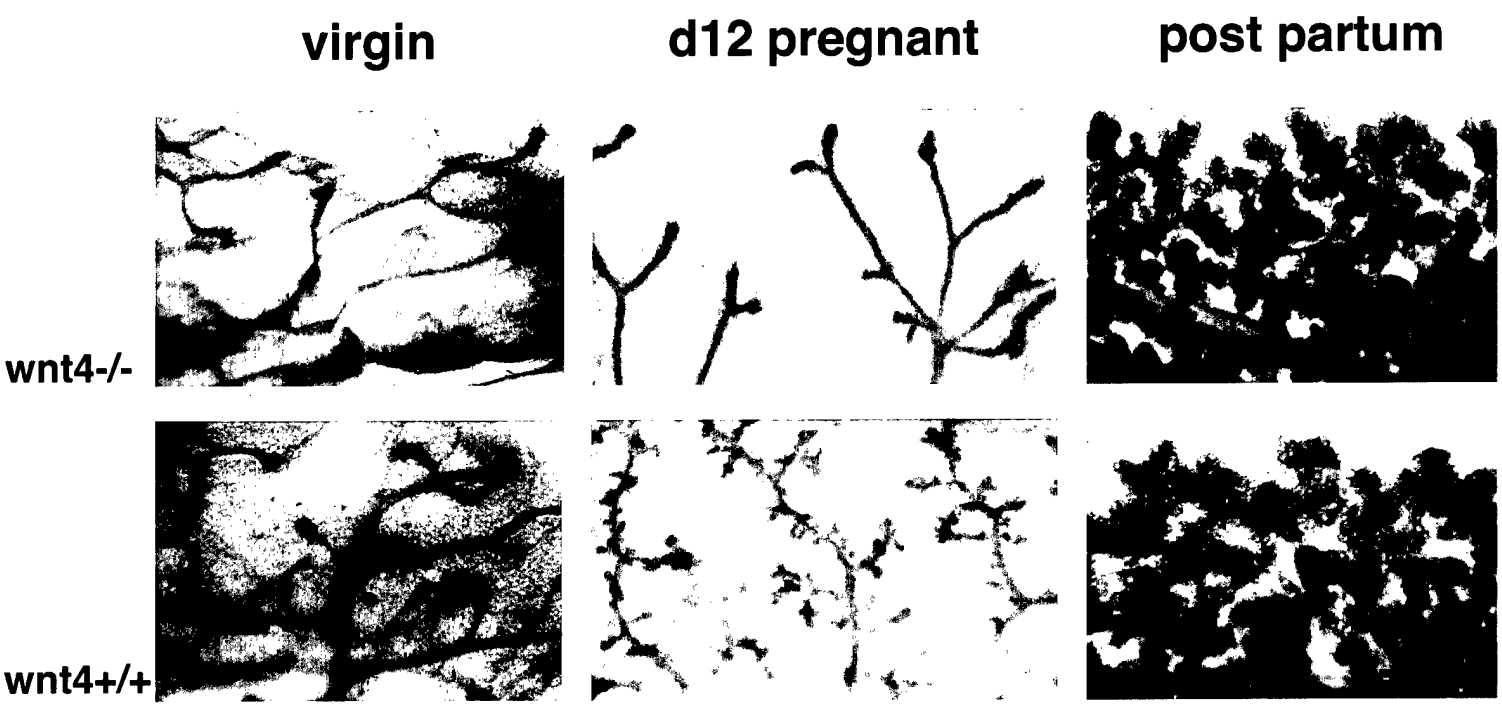


Figure 2



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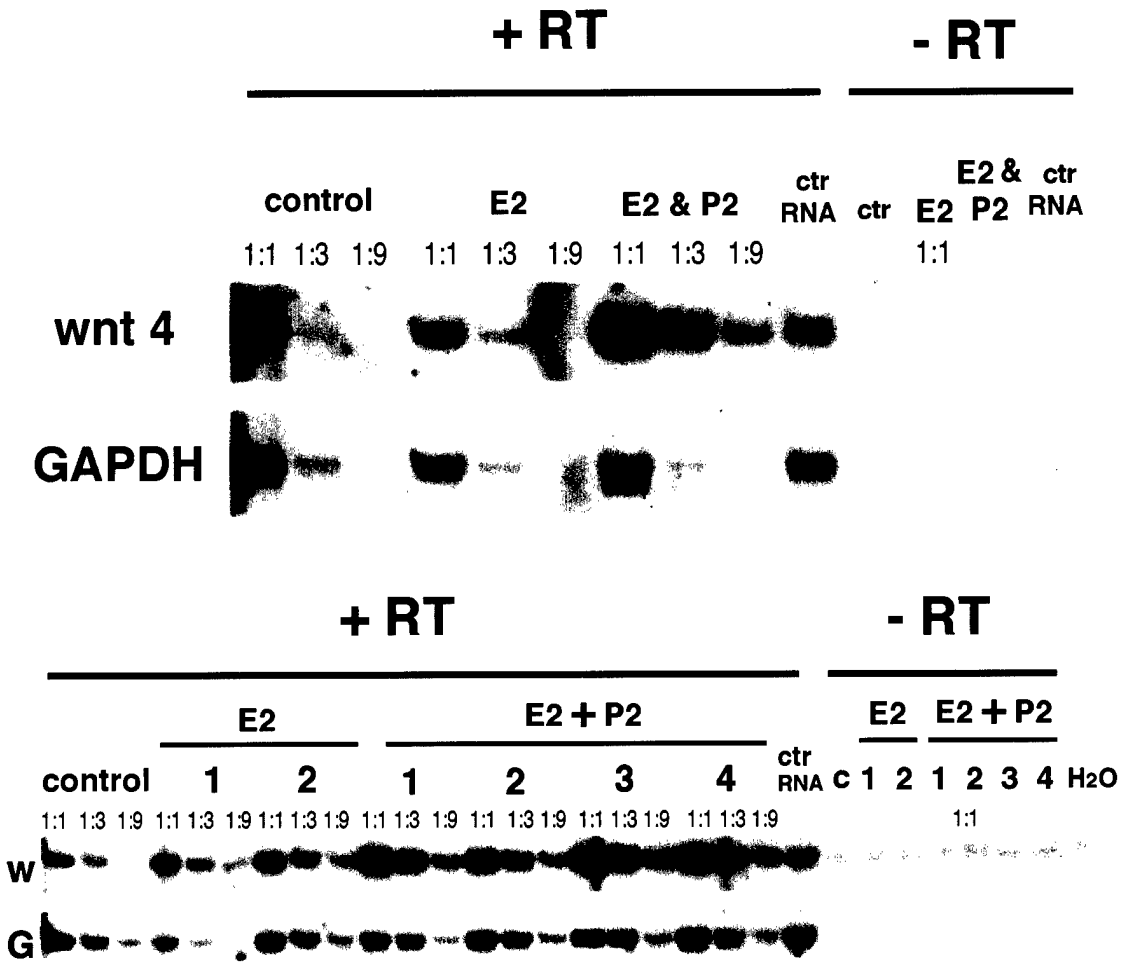
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Figure 4A

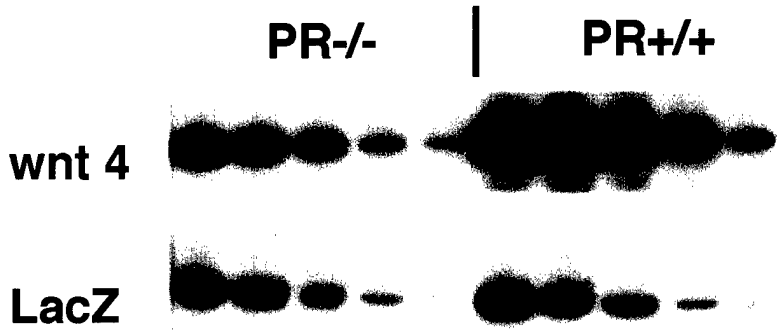

Figure 4B

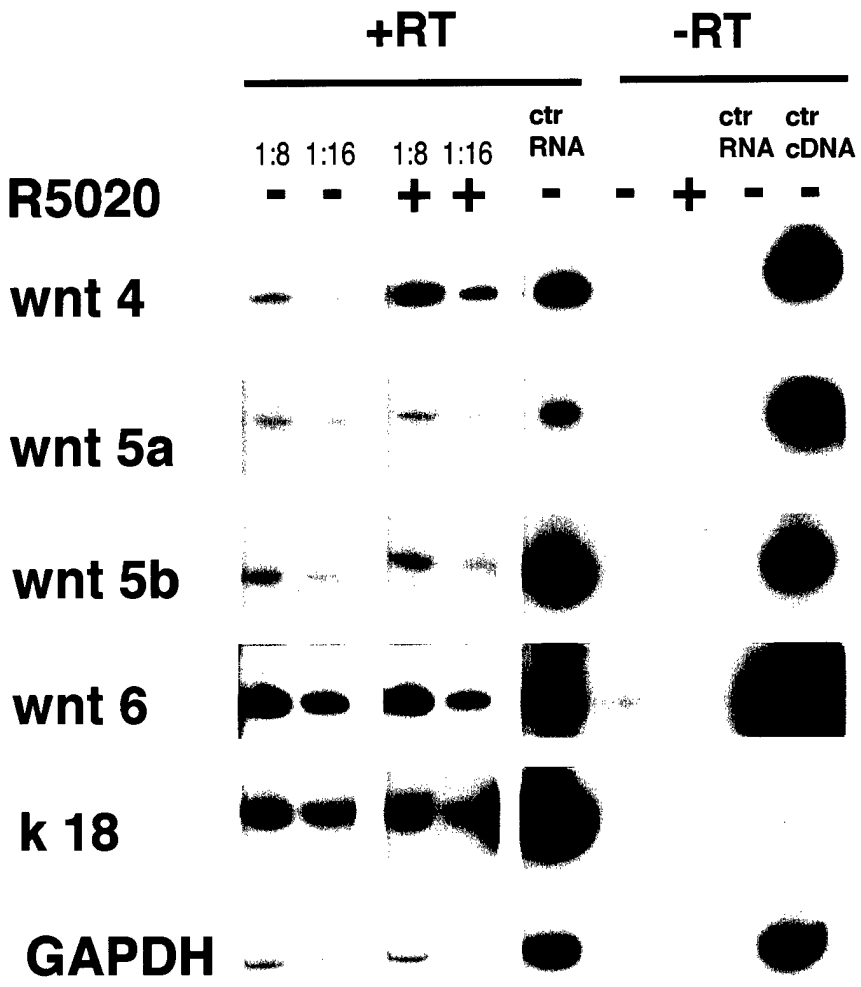
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Figure 4C